SOME FUNCTIONS OF THE URINARY BLADDER IN A CRAB

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The antennary glands of crabs generally are ineffective as organs of osmoregulation inasmuch as the urine they produce remains isosmotic with the blood under conditions of hypo- or hyperosmotic stress. On the other hand, as indicated in the reviews by Lockwood (1962) and Potts and Parry (1964) the probable primary function of these renal organs is ionic regulation. Of particular interest is the high concentration of Mg^{++} attained in the urine of crabs during immersion in hypersaline water.

Prosser *et al.* (1955) demonstrated in the shore crab, *Pachygrapsus crassipes,* dramatic increases in urine Mg^{++} but decreases in urine Na^+ as the environmental salinity increased. This, they attributed to competition between Na^+ and Mg^{++} for transport across the membranes of the antennary gland, Mg^{++} prevailing. Green *et al.* (1959) observing a similar phenomenon in two species of *Uca* also suggested that Mg^{++} and Na^+ compete for transport and that active movement of Na^+ is reduced by such competition under the high Mg^{++} load found in hypersaline water. These authors as well as Riegel and Lockwood (1961), who observed the phenomenon in *Carcinus,* considered but rejected direct Mg^{++}-Na^+ exchange as the mechanism of concentrating Mg^{++} at the expense of Na^+. Whatever the mechanism, the phenomenon seems to be common in crabs (Gross, 1964; Gross *et al.,* 1966).

Gross (1964), examining a series of crabs from aquatic, amphibious and terrestrial modes of life, revealed that animals showing high degrees of terrestrialness tended to concentrate Mg^{++} more highly in the urine than the more aquatic crabs. An exception was the terrestrial *Gecarcinus lateralis* which is the only brachyuran crab examined to date incapable of concentrating urine Mg^{++} at the expense of Na^+. Still, it was shown that high urine Mg^{++} does not necessarily reflect strong Mg^{++} regulation in the blood. For example, the urine Mg^{++} of the amphibious *Uca* was more than three-fold that of the aquatic *Cancer*, yet the blood Mg^{++} concentrations of these two species were about the same.

Gross and Marshall (1960) demonstrated that the concentration of Mg^{++} in the urine of *Pachygrapsus* is independent of the Mg^{++} influx and in some way a function of the osmotic concentration of the external medium. This phenomenon also was demonstrated in *Cardisoma carnifex, Varuna litterata* and *Sesarma mcinerti* (Gross *et al.,* 1966).

The above described phenomena lead to the following questions: (1) What is the relationship between Mg^{++} concentration in the urine of a crab and the amount of Mg^{++} it excretes? (2) By what means does a crab immersed in a Mg^{++}-free medium of high salinity concentrate Mg^{++} in its urine? (3) By what means does the urine Na^+ concentration become reduced as the urine Mg^{++} concentration elevates when the animal is transferred from dilute to concentrated sea water?

The present investigation produces evidence that Mg^{++} concentration in the urine depends on the relative length of time the latter is held in the bladder. Mg^{++} is transported across the walls of the bladder into the urine at different rates depending on the blood Mg^{++} concentration and a direct exchange with Na^+ can take place which effects movement of water between blood and urine.

Materials and Methods

The shore crab, *Pachygrapsus crassipes* Randall, which is a known hypo- and hyperosmotic regulator (Jones, 1941; Prosser *et al.*, 1955; Gross, 1957) was collected at Laguna, California, and maintained in the laboratory at 15° C. in 100% artificial sea water made from the Utility Chemical Company Seven-Seas Marine Mix. Only intermolt crabs larger than 15 grams were used in the experiments. A salinity of 34.3‰ was considered to be 100% sea water. This contained the following cation concentrations: Na^+, 455 mM/l.; K^+, 11.5 mM/l.; Ca^{++}, 14.2 mM/l. and Mg^{++}, 55.5 mM/l. Different concentrations of sea water were attained by varying the amounts of water added to these salts. $MgCl_2$ was added to test media where the Mg^{++} concentration was to be higher than normal. Also, artificial sea water for experiments concerned with Mg^{++} depletion was made up using the proportions of Na^+, K^+, Ca^{++}, Mg^{++}, Cl^- and $SO_4^=$ given in the tables of Barnes (1954) with the pH adjusted to 8.0. Na^+ was substituted for Mg^{++} when the latter was deleted.

Perfusion fluid used to simulate plasma and/or primary urine contained the following concentrations of ions: Na^+, 483 mM/l.; K^+, 7 mM/l.; Mg^{++}, 10 mM/l.; Ca^{++}, 15 mM/l., Cl^-, 520 mM/l. and $SO_4^=$ 10 mM/l. This approximates the blood cation and osmotic concentration of *Pachygrapsus* when immersed in normal sea water (Gross, 1959; 1964). The Cl^- concentration approximates the mean blood concentration (517 mM/l., S.D., 11.3) of 6 crabs taken from 100% sea water. Concentrations of $SO_4^=$ were estimated by difference assuming Cl^- and $SO_4^=$ as the only anions and considering electro-chemical balance. Hereinafter isosmotic perfusion fluid will mean a solution made up of the above proportions but adjusted by water content to be approximately isosmotic with the blood for crabs immersed in a particular salinity. Blood osmotic concentrations for crabs immersed in different salinities are given by Gross (1957; 1964).

Immersion experiments were conducted using approximately 400 ml. of medium which was sufficient to assure complete immersion.

Osmotic concentrations of media and body fluids were determined by means of a Mechrolab, vapor pressure osmometer. Na^+ and K^+ were determined by flame photometry; Ca^{++} and Mg^{++} by ethylene diamine tetra acetic acid (EDTA) titration as previously described (Gross, 1959; Gross *et al.*, 1966); Cl^- by the method of Schales and Schales (1941); inulin was determined by the resorcinol method of Schreiner (1950).

In the range of normal sea water osmotic concentrations could be measured within a 1% error, Na^+, about 2%, K^+, less than 10%, Ca^{++} and Mg^{++} less than 6% and microsamples of Cl^- to less than 10%. Inulin could be measured with less than a 7% range of error.

Blood was extracted by puncturing the arthrodial membranes at the bases of the walking legs with a glass pipette. Urine was removed from the nephropore by

means of a fine glass cannula.   Since urine is clear and blood turbid, any contamination of urine with blood could easily be detected.   Doubtful samples were discarded.

Results

It has been shown that when *Pachygrapsus* is immersed in 100% and 150% sea water the urine Mg^{++} concentrations averaged 118 mM/l. and 204 mM/l., respectively, the corresponding urine concentration/blood concentration (U/B) values for Mg^{++} being 13.6 and 15.4 (Gross, 1959).   The following experiment therefore was performed to show the role of water withdrawal in achieving the above urine Mg^{++} concentrations and U/B values.   The bladders of crabs which had been immersed in 100% or 158% sea water were drained, and the animals were injected with about 0.1 ml. of an isosmotic perfusion of fluid containing approximately 6% inulin.   The crabs then were reimmersed in the media from which they were taken, and after 6 or 48 hours the urine and blood were sampled for inulin analysis. Another group taken from 100% sea water was also thus treated but was kept out of water rather than reimmersed.   Thus, it can be seen that the U/B values (Table I) were so low that water withdrawal cannot be a major factor in effecting

Table I

Inulin U/B values of Pachygrapsus

	6 hours exposure			48 hours exposure		
	No.	Mean	S.D.	No.	Mean	S.D.
100% sea water	13	1.11	0.13	10	1.92	1.29
158% sea water	10	1.16	0.24	13	1.52	0.65
Air	7	1.09	0.17	5	1.44	0.12

high Mg^{++} concentrations or U/B values.   Of the mean U/B values presented in Table I only those for crabs immersed in 158% sea water or kept in air for 48 hours are significantly different from unity ($P < 0.02$).   The means for the three 6-hour experiments are not significantly different.   The means for the three 48-hour experiments are not significantly different, and there is no significant difference between 6- and 48-hour treatments for either salinity.   The difference in urine Mg^{++} concentration in animals immersed in normal sea water compared to those immersed in hypersaline water therefore cannot be achieved by differences in water withdrawal from the urine.   Further reference will be made to data in Table I later.

After 24 hours immersion in 158% Mg^{++}-free sea water, the urine Mg^{++} of 15 crabs averaged 235 mM/l. (S.D., 117) whereas the mean urine Mg^{++} of 18 crabs immersed for 24 hours in 50% sea water containing 65 mM/l. of Mg^{++} was only 20.5 mM/l. (S.D., 8.90).   These data, which confirm the observations of Gross and Marshall (1960), clearly show that the ability of *Pachygrapsus* to concentrate Mg^{++} in the urine is neither a function of Mg^{++} influx nor the concentration of Mg^{++} in the medium.

Figure 1 illustrates the frequency distribution for the urine Mg^{++} concentrations of 51 crabs sampled in the field where only normal sea water was available. Figure 2 shows urine $Mg^{++} + Ca^{++}$ concentrations of crabs totally immersed in a running sea water aquarium containing 100% sea water. Small quantities of urine ($\sim$20 μl.) were periodically sampled from the same nephropore of individual crabs, 10 μl. of which were analyzed for $Mg^{++} + Ca^{++}$. Ca^{++} was not determined because of the small sample size. There it can be seen that the concentration of $Mg^{++} + Ca^{++}$ varies tremendously with time, and since urine Ca^{++} is relatively constant in concentration (approximately 20 mM/l.) with little variance (Gross, 1959), the large fluctuations in Figure 2 can be attributed to Mg^{++}. This might suggest that the
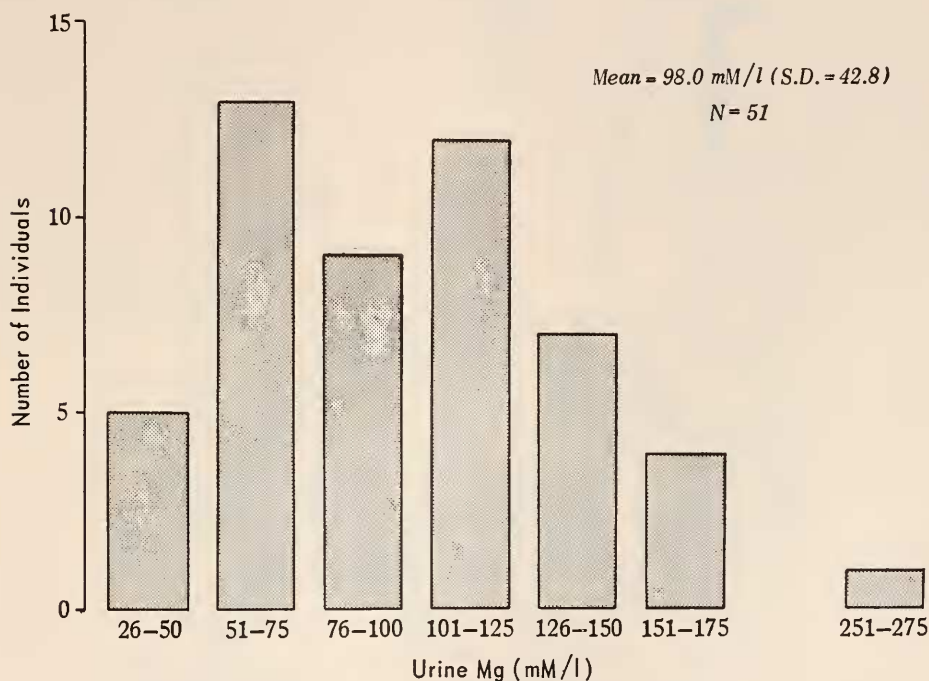

FIGURE 1. Frequency distribution for urine Mg^{++} concentrations of crabs sampled in the field where only 100% sea water was available.

Mg^{++}-transporting mechanism fluctuates in its rate of activity. However, another possibility is that the urinary bladder itself has a transporting function with respect to Mg^{++}. That is, urine entering the bladder from the labyrinth is relatively low in Mg^{++}. If the urine were held in the bladder for a prolonged period, sufficient time would permit elevation of the Mg^{++} concentration. Following bladder evacuation, then, the urine Mg^{++} should be low. When a hypo-regulating crab is immersed in hypersaline media, the water influx would be slow, the bladder would be evacuated with low frequency and urine would be held in the bladder sufficiently long to permit accumulation of Mg^{++}. On the other hand, in low salinities, water influx would be rapid in a hyper-regulating crab, evacuation of the bladder would be

frequent and no time permitted for Mg^{++} accumulation. Such a model would explain the high concentration of urine Mg^{++} for crabs immersed in 158% Mg^{++}-free sea water and low Mg^{++} concentration in urine of crabs immersed in 50% sea water containing high Mg^{++}. This would also explain the fluctuations in urine Mg^{++} shown in Figure 2. That is, low Mg^{++} concentrations would follow bladder evacuation and high Mg^{++} concentrations would precede evacuation.
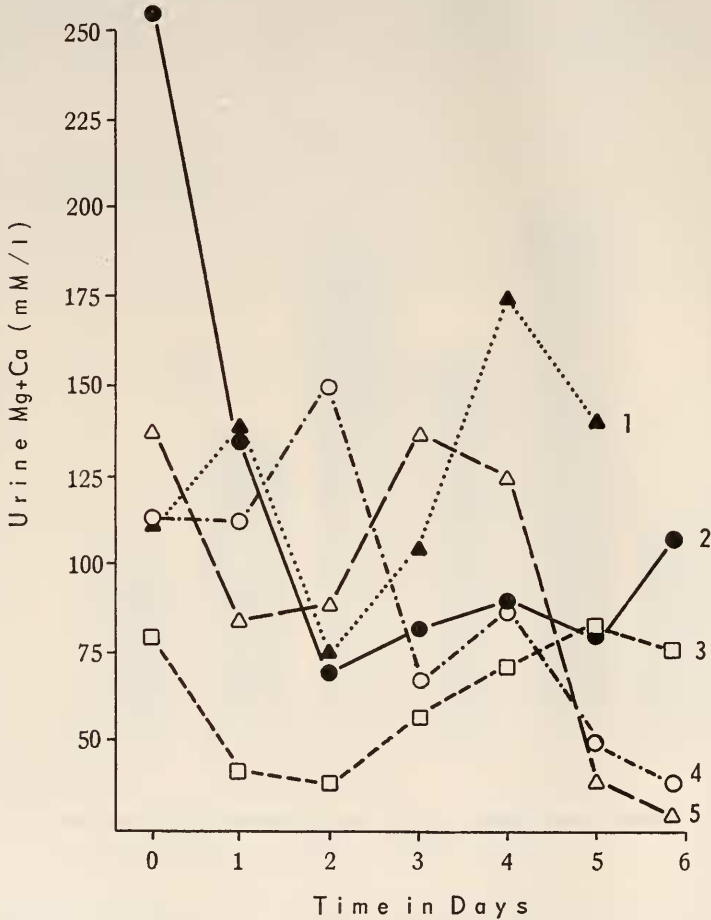

FIGURE 2. Fluctuations in the concentration of urine Mg^{++} + Ca^{++} of individual crabs immersed in 100% sea water. Each symbol connected by line represents history of individual crab.

In order to test this model, one of the paired bladders of a crab immersed in 100% sea water was evacuated; the crab was then reimmersed in 100% sea water and after a given period, urine from the same bladder was sampled for Mg^{++} analysis. Thus, for 17 crabs reimmersed 2–24 hours, the mean Mg^{++} concentration was 69 mM/l. (S.D., 55) and for 16 crabs reimmersed 48–96 hours, the mean Mg^{++} concentration was 165 mM/l. (S.D., 99), the two means being significantly
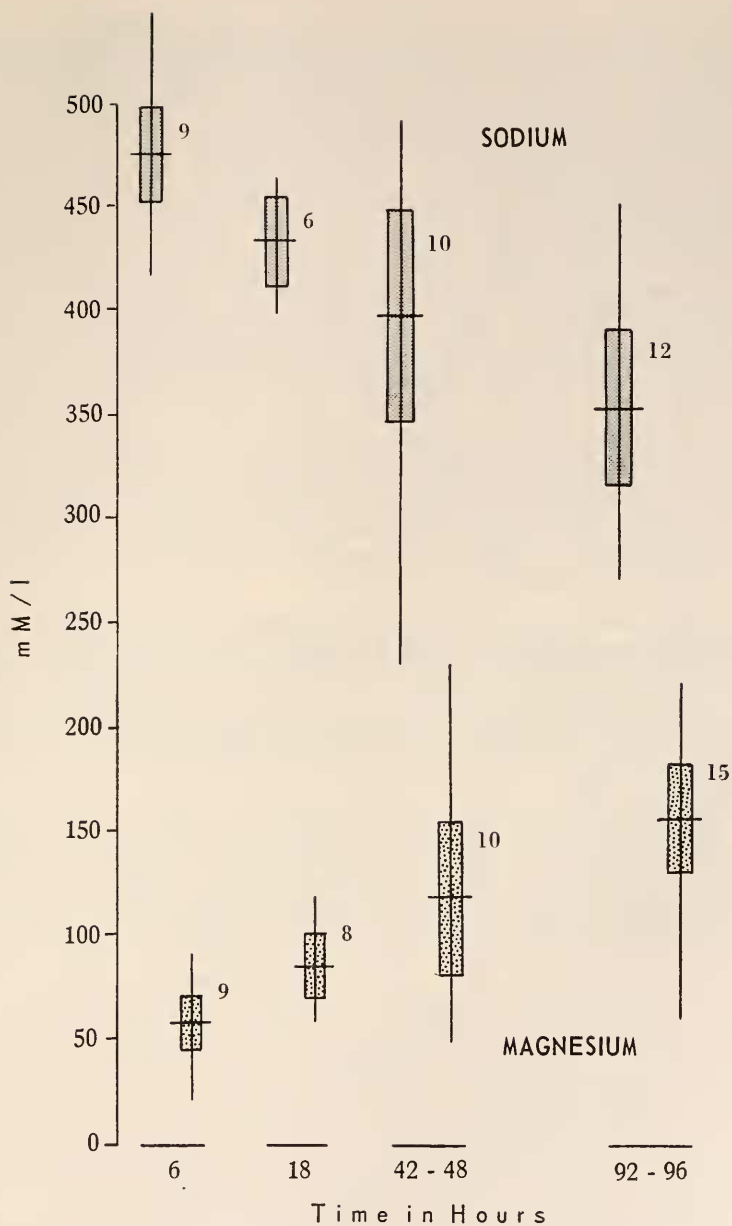
FIGURE 3. Decreases in urine Na^+ concentration accompanying increases in urine Mg^{++} concentration in crabs immersed in 100% sea water as a function of time after bladder evacuation. Mean is represented by horizontal line; range by vertical line and twice the standard error on either side of the mean by the rectangle. Numerals indicate number of cases.

different $(P < 0.01)$. If it is assumed (in the above experiment) that crabs immersed for the longer periods also retain (on the average) urine in the bladder for longer periods, these data suggest that urine first entering the bladder is relatively low in Mg^{++} concentration, and as it is held in the bladder Mg^{++} is added to it.

Figure 3 illustrates an experiment which lends support to this suggestion. The bladder of a crab that had been immersed in 100% sea water was drained and flushed with perfusion fluid in order to remove high concentrations of residual Mg^{++}. After flushing the bladder, the fluid was removed and the crab with the empty bladder was reimmersed in 100% sea water. Following a given period of immersion, the urine was removed from the same bladder and analyzed for Mg^{++} and Na^+. As can be seen in Figure 3, 6 hours after reimmersion, the mean urine Mg^{++} concentration was low and the mean urine Na^+ concentration was high. As the immersion period increased and presumably the average period of urine retention increased, the mean urine concentration of Mg^{++} increased and the mean urine concentration of Na^+ decreased. For both urine Na^+ and Mg^{++}, the 92–96-hour group (mean) was greatly different from the 6-hour group $(P < 0.001)$. It should be pointed out that urine samples taken 6 hours following bladder evacuation are also isosmotic with the blood. Thus, 15 crabs, which included 5 immersed in 100% sea water, 5 immersed in 158% sea water and 5 kept out of the water, had an osmotic U/B mean of 1.007 (S.D., 0.0176).

The possibility was considered that the empty bladder encouraged a rapid surge of fluid through renal organ and that insufficient time was allowed for Mg^{++} to concentrate in the urine before entering the bladder. As the bladder filled, the flow of urine through the labyrinth, for example, would be retarded and the subsequent urine entering the bladder would be relatively high in Mg^{++}. An experiment therefore was conducted showing that increases in Mg^{++} occur in the urine with time when the bladder is full.

Gross and Marshall (1960) gave evidence that *Pachygrapsus* does not lose urine when kept out of the water. The following preliminary experiment was conducted to demonstrate that fluid introduced into the bladder after artificial evacuation will be held in the bladder while the animal is kept out of the water. Urine from one bladder of the crab was emptied, flushed with an isosmotic solution colored with indigo carmine, emptied again and refilled. If there was no immediate sign of leakage due to injury of the nephropore, the dried animal was placed in a dry plastic container, the floor of which was covered with several layers of white absorbent tissue paper. In such a situation any loss of "urine" would stain the white paper. Of 20 animals thus tested using the following isosmotic solutions: (a) perfusion fluid for 24 hours (10 crabs); (b) NaCl for 3 hours (7 crabs) and (c) $MgCl_2$ for 3 hours (3 crabs) only one (NaCl) lost "urine" but this still had dye in the "urine" remaining in the bladder, indicating that only part of the introduced fluid leaked out. All other crabs retained sufficient color in the bladder fluid until the end of the experiment to have stained the white paper had fluid been lost. Still, after 24 hours the bladder fluid had lost considerable color, indicating absorption of the dye. Thus, such an experiment would be of little value if continued for more than one day. Nevertheless, the probability is high that isosmotic fluids introduced into an empty bladder will remain there for at least 24 hours if the crab is kept out of the water. It should also be noted that when dye is introduced

into one of the paired bladders, it does not appear in the other side, indicating that the bladders are isolated from each other.

Next, bladders of crabs removed from 100% sea water were evacuated, rinsed and filled with the above-described perfusion fluid containing 10 mM/l. of Mg^{++}. The animals were placed in dry containers and after selected periods the bladder fluid was sampled and analyzed for Mg^{++}. The bladder fluid of 10 animals so treated averaged 33.2 mM/l. (S.D., 11.0) for Mg^{++} 1–3 hours after introduction of the fluid, whereas the bladder fluid of 8 crabs averaged 64.5 mM/l. (S.D., 27.5) after 28–48 hours. These two groups are significantly different ($P < 0.01$) and only part of this difference could be caused by water withdrawal (Table I). Thus, urine Mg^{++} concentrates with time in a full bladder. This is interpreted to mean that the walls of the bladder transport Mg^{++} into the urine and prolonged retention of urine in the bladder results in the attainment of high Mg^{++} concentrations in the urine.

Evidence has been produced that *Pachygrapsus* does not lose urine when out of the water. On the other hand, when the bladder is emptied, it will readily fill even though the crab is not immersed. Substantial urine samples can be extracted from the bladders of most "dry" crabs 6 hours after bladder evacuation. Twenty-four hours after emptying, the bladders of crabs kept in dry situations seem as full as those of immersed crabs.

Figure 4 illustrates how crabs placed in dry containers with empty bladders (previously rinsed with isosmotic perfusion fluid) concentrate Mg^{++} in the urine with time at the expense of Na$^+$. As shown for the immersion experiments, urine Mg^{++} increases with time after bladder evacuation, but Na$^+$ decreases with time.

Now if the period of time urine is held in the bladder dictates the concentration of urine Mg^{++}, then blocking the nephropore to prevent urine release should result in an increase in the Mg^{++} concentration of the urine. Thus, one of the paired nephropores of *Pachygrapsus* was blocked with epoxy cement and after the animal was immersed in 50% sea water for 24 hours, urine from both blocked and unblocked sides was extracted and analyzed for Mg^{++}. In every case (12) urine from the blocked bladder was higher in Mg^{++} than urine from the unblocked bladder, the mean ratio, blocked/unblocked being 2.63 (S.D., 1.19) which is significantly different from unity ($P < 0.001$).

Four lines of evidence have been presented indicating that the bladder of *Pachygrapsus* transports Mg^{++} from the blood into the urine, thus increasing the concentration of Mg^{++} in the urine with time as it is retained in the bladder far beyond that which could be caused by water withdrawal (Table I): (1) Crabs immersed with empty bladders show increased urine Mg^{++} concentrations with time; (2) when perfusion fluid is substituted for urine in the bladder, the Mg^{++} concentration of the bladder fluid increases with the period the crabs are kept out of the water; (3) when crabs with emptied bladders are kept out of the water, fluid low in Mg^{++} fills the bladder, but with time the concentration of urine Mg^{++} increases; (4) when urine from blocked and unblocked bladders of the same immersed crab are compared, urine from the blocked side is higher in Mg^{++} than urine from the unblocked side.

Since the phenomenon illustrated in Figures 3 and 4 suggests a direct Mg^{++}-Na$^+$ exchange, isosmotic solutions of NaCl or MgCl$_2$ were substituted for urine in

bladders of crabs kept out of water. One bladder of each crab first was evacuated of urine, rinsed twice with isosmotic test solution and then filled with a volume of test solution which approximated the volume of urine removed. After the crab was kept for a given period in air, the test solution was removed from the bladder and analyzed for Na^+ and Mg^{++}. In this way Na^+ and Mg^{++} concentration changes could
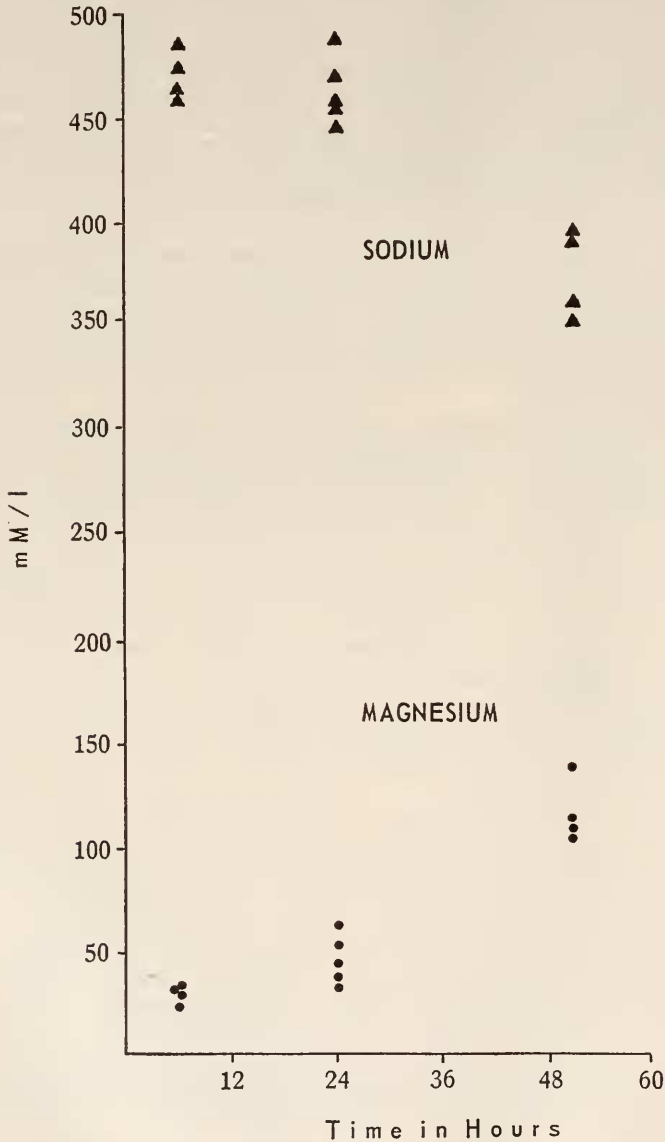

FIGURE 4. Decreases in urine Na^+ concentration accompanying increases in urine Mg^{++} concentration in crabs kept out of the water as a function of time after bladder evacuation. Triangles represent urine Na^+; circles urine Mg^{++}. Each point represents a single determination.

be measured in the bladder fluid and assuming constancy of bladder fluid volume, this information could give the relative number of Na^+ ions exchanged for Mg^{++} ions. Table II includes all cases of this experiment where there was no immediate indication of leakage from the nephropore due to injury and where there was sufficient concentration change of both ions to be measured quantitatively. Thus, it can be seen for both NaCl and $MgCl_2$ that whenever there was a gain in Mg^{++} concentration in the bladder fluid there was a loss in Na^+ concentration and *vice versa*. The

TABLE II

Na^+–Mg^{++} exchange through bladder wall of Pachygrapsus

Bladder solution	Spec. no.	Na^+ change (mM/l.)	Mg^{++} change (mM/l.)	$\frac{Na^+ \text{ change}}{Mg^{++} \text{ change}}$	Time hrs.
560 mM/l. NaCl		−	+		
	1	116	82	1.42	1.0
	2	80	44	1.82	1.5
	3	100	55	1.82	2.0
	4	126	87	1.45	3.0
	5	80	31	2.58	3.0
	6	47	24	1.96	3.0
	7	64	36	1.78	3.0
	8	56	31	1.81	3.0
	9	81	69	1.17	18.0
	10	79	48	1.64	18.0
	11	96	72	1.33	18.0
	12	78	69	1.13	18.0
	13	48	34	1.41	18.0
	14	130	74	1.76	19.0
360 mM/l. MgCl$_2$		+	−		
	15	52	23	2.26	1.0
	16	265	218	1.22	1.0
	17	235	154	1.53	1.0
	18	218	127	1.72	1.0
	19	420	264	1.59	1.0
	20	362	233	1.55	1.0
	21	60	35	1.71	1.5
	22	322	221	1.46	2.0
	23	241	155	1.55	19.0
	24	358	219	1.63	19.0
Mean				1.64	
S.D.				0.33	

mean ratio, Na^+ concentration change/Mg^{++} concentration change, was 1.64. Now, assuming no net anion movements, for every divalent Mg^{++} ion transported, two monovalent Na^+ ions should be exchanged. Chloride constitutes most of the urine anions because the urine for ten crabs removed from normal sea water had a mean osmotic concentration of 1040 mOsm/l. (S.D., 12.6) and a mean urine chloride of 516 mM/l. (S.D., 27.2). Green *et al.* (1959) stated that if Na^+-Mg^{++} exchange occurred, the Na^+ change/Mg^{++} change should be 2. However, the loss of two Na^+

ions for every Mg^{++} ion gained would reduce the osmotic concentration of the bladder fluid. Yet isosmotic NaCl solution introduced into empty bladders of 8 crabs remained essentially isosmotic for three hours when the animals were kept out of the water, the mean osmotic urine/blood value being 1.01 (S.D., 0.018). Since the Na^+-Mg^{++} exchange would reduce the urine osmotic concentration, water must move to effect the isosmotic condition between blood and urine. Therefore, the observed Na^+ change/Mg^{++} change should be related to the isotonic coefficients for NaCl and $MgCl_2$ which were empirically determined to be 1.8 and 2.8, respectively, at the initial test concentration (Table II). Therefore, the Mg^{++} concentration change $\times$ 2.8 = Na^+ concentration change $\times$ 1.8, or Na^+ concentration change/Mg^{++} concentration change = 2.8/1.8 = 1.56, a value which closely approximates the observed value, 1.64 (Table II). This close agreement is interpreted as evidence that a direct Na^+-Mg^{++} exchange can indeed take place. It also seems that such an exchange can take place in either direction across the membranes of the bladder. This in turn suggests relative impermeability of those membranes to chloride.

However, when isosmotic perfusion fluid was used instead of NaCl or $MgCl_2$, the mean Na^+ concentration change/Mg^{++} concentration change for 6 crabs after 24 hours was only 1.15 (S.D., 0.14) which is significantly less than the mean 1.64 given in Table II ($P < 0.001$). The longer test period for perfusion fluid was necessary to permit a measurable cation change. A possible reason for these conflicting results will be given below.

The question now may be raised as to the dependence of Mg^{++} transport on Na^+ active transport. An attempt therefore was made to block active transport of Na^+ from the lumen of the bladder into the hemocoele by ouabain which is known to inhibit Na^+ transport (Judah and Ahmed, 1964). One bladder of the crab was drained of urine, rinsed with isosmotic perfusion fluid containing 10 mM/l. Mg^{++} and refilled with the same perfusion fluid containing 5×10^{-4} or 10^{-3} M ouabain. The crab then was placed in a dry container for 24 hours after which time the bladder was drained again and urine analyzed for Mg^{++}. The mean urine Mg^{++} concentration for 14 crabs thus treated was 120 mM/l. (S.D., 88.5). Even though low activity of the crabs indicated that the ouabain had diffused into the blood and was present on both sides of the bladder membrane, it obviously did not prevent accumulation of Mg^{++} in the bladder fluid.

The mean urine Na^+ concentration of 13 crabs after this treatment was 460 mM/l. (S.D., 27.4) which was not significantly different from the initial Na^+ concentration (483 mM/l.). However, the highest urine Mg^{++} concentrations were accompanied by the lowest Na^+ concentrations, so it is believed that either Na^+ movement, in this case, is a passive process or ouabain was ineffective in blocking the Na^+ transport mechanism in all cases. Nevertheless, there is no evidence that Mg^{++} secretion is coupled to the Na^+ transport mechanism, but there is further evidence that Mg^{++} accumulates in a full bladder with time. It might be that the Mg^{++} ion can exchange for any other cation, but since Na^+ is the dominant one, loss of Ca^{++} or K^+ from the urine in exchange for Mg^{++} could not be detected by the methods used in this investigation. Exploratory experiments where the bladder was filled with a perfusion fluid in which choline was substituted for Na^+ showed that Mg^{++} was concentrated in the bladder fluid after 24 hours. However, Na^+ was

also high in the bladder fluid and had obviously diffused from the blood down the steep gradient. Thus, it was not determined whether or not Mg^{++} was exchanged for choline.

In view of the above findings, there can be little doubt that Mg^{++} is concentrated in the urine by the bladder and that the Mg^{++} concentration is a function of the time urine is retained in the bladder. However, evidence was produced in the following experiments that the rate of Mg^{++} transport into the bladder is higher when the crab is in hypersaline water than when in normal sea water. That is, the amount of Mg^{++} entering the bladder 6 hours following evacuation is greater in crabs immersed in hypersaline water than those immersed in normal sea water.

In Group One (10 animals), the bladder of the crab was drained and flushed with isosmotic perfusion fluid, then drained again. The crab was reimmersed in 100% sea water and after 6 hours, urine from the same bladder was sampled for Mg^{++} analysis.

In Group Two (17 animals), the crab was first immersed in 158% sea water for 18–24 hours; the bladder was drained, rinsed with isosmotic perfusion fluid containing about 15 mM/l. Mg^{++} and reimmersed in 158% sea water for an additional 6 hours. After this period the urine was completely drained from the same bladder for Mg^{++} analysis.

In Group Three (20 animals), the crab was first immersed in 158% sea water for 18 hours; the bladder was drained, rinsed with isosmotic perfusion fluid containing about 15 mM/l. Mg^{++} and reimmersed in 158% Mg^{++}-free sea water for an additional 6 hours. The urine was then extracted for Mg^{++} analysis.

Thus, the 6-hour urine sample for crabs immersed in 100% sea water (Group One) averaged 54.5 mM/l. (S.D., 20.1), whereas for crabs immersed in 158% sea water (Group Two) the mean urine Mg^{++} was 113 mM/l. (S.D., 70.0). A second 6-hour sample was taken from Group Two (*i.e.*, 12 hours after rinsing of the bladder and reimmersion in 158% sea water) and the mean urine Mg^{++} then was 100 mM/l. (S.D., 48.5), indicating that the difference between 6-hour urine Mg^{++} in 100% and 158% sea water treatments is not merely a matter of residual Mg^{++} in the bladder of crabs immersed in 158% sea water for 18 hours. The mean urine Mg^{++} for Group Three which had been immersed for 6 hours in 158% Mg^{++}-free sea water was 120 mM/l. (S.D., 63.0). This mean as well as those for Group Two are significantly larger than the mean for Group One ($P < 0.01$).

Inulin U/B values (Table I) indicate that water withdrawal from urine is no greater for crabs immersed in 158% sea water than for those immersed in 100% sea water. Therefore, the different urine Mg^{++} concentrations produced during the 6-hour period by crabs in the two salinities cannot be explained on the basis of water withdrawal.

Inasmuch as the 6-hour urine sample for crabs immersed in 158% Mg^{++}-free sea water (Group Three) was equally as high in Mg^{++} as that of crabs immersed in 158% sea water containing high Mg^{++}, there is evidence that Mg^{++} transport from blood to urine is independent of the Mg^{++} concentration in the external medium and in turn independent of the Mg^{++} influx from the external medium to crab.

The higher concentrations of urine Mg^{++} observed above in crabs immersed for 6 hours in hypersalinities over those immersed for 6 hours in 100% sea water may indicate that: (1) the rate of Mg^{++} transport from blood into urine is higher

when the crab is immersed in hypersaline water than when it is immersed in normal sea water; (2) the rate of Mg^{++} transport is constant, but the volume of primary urine formed is smaller after 6 hours in 158% sea water than after 6 hours in 100% sea water, thus effecting a higher concentration of Mg^{++} in the urine while accumulating the same amout of Mg^{++}, and (3) there is reduced primary urine accompanied by increased Mg^{++} transport for crabs immersed in hypersalinities compared to those in normal salinity. If primary urine were formed by filtration, its rate of formation would be expected to be slower when the crab was in hypersalinities than when in normal salinity. Lockwood (1962) discusses the possibility of renal filtration among crustaceans in general. Kirschner and Wagner (1965) produce evidence of filtration in a fresh-water crayfish. To date, no reliable values have been obtained on the rate of primary urine production in *Pachygrapsus* for any treatment. However, evidence will be produced below that there is actually a

TABLE III

Elements influencing the concentration of Mg^{++} in urine

| | Group A | | | Group B | | | Group C | | |
| | 50% sea water with 65 mM/l. of Mg^{++} for 18 hours to 100% sea water (6 hours) | | | 158% sea water with 59 mM/l. of Mg^{++} for 18 hours to 100% sea water (6 hours) | | | 158% sea water with 82 mM/l. of Mg^{++} for 24 hours to 158% Mg^{++}-free sea water (6 hours) | | |
	No.	Mean	S.D.	No.	Mean	S.D.	No.	Mean	S.D.
Urine Mg^{++} (mM/l.)	8	29.9	13.2	14	60.6	39.7	11	123.8	66.6
Blood Mg^{++} (mM/l.)	11	12.4	2.11	15	14.8	5.68	12	14.5	1.75
Blood osmotic concentration (% sea water)	12	93.7	4.60	15	115.5	2.51	12	135.0	6.97

higher rate of Mg^{++} transport for crabs in hypersaline water than for those immersed in 100% sea water.

Assuming for the moment such an increase in Mg^{++} transport does occur, then any of the following or combination of the following could be responsible for triggering the accelerated rate of such transport from blood into the bladder: (1) direction of passive water flux between animal and medium; (2) osmotic concentration of the external medium; (3) osmotic concentration of the blood, and (4) Mg^{++} concentration in the blood. Mg^{++} concentration in the medium and Mg^{++} influx already have been ruled out as triggering stimuli.

The experiment summarized in Table III was designed to test the direction of passive water flux and blood osmotic concentration as factors for controlling the rate of Mg^{++} transport when the blood Mg^{++} and osmotic concentration of the medium were held constant. Thus, Group A was immersed for 18 hours in a medium equivalent to 50% sea water in osmotic concentration, but containing 65 mM/l. of Mg^{++} which is about twice that present in 50% natural sea water. After 18 hours immersion one bladder of the crab was emptied, rinsed with isosmotic

perfusion fluid and emptied again. The crab then was immersed in 100% sea water for a period of 6 hours, after which time urine was removed from the same bladder for Mg^{++} analysis.

Group B was immersed for 18 hours in a medium equivalent to 158% sea water in osmotic concentration but containing 59 mM/l. of Mg^{++} which is about that found in 100% natural sea water and comparable to the concentration of Mg^{++} in the medium for Group A (above). After 18 hours, one bladder of the crab was emptied, rinsed with isosmotic perfusion fluid, emptied again and reimmersed in 100% sea water for 6 hours. After this period, the same bladder was drained and the urine analyzed for Mg^{++}.

For the second step of this experiment, that is, immersion in 100% sea water, the blood of Group A was osmotically less concentrated than the medium and the blood of Group B was osmotically more concentrated than the medium (Table III). Thus, with respect to the direction of passive water flux, Group A was simulating hypo-regulation (passive water efflux) and Group B hyper-regulation (passive water influx) which normally, when observed in crabs in high and low salinities, are accompanied by high and low urine Mg^{++} concentrations, respectively. If, then, the direction of passive water flux were a major factor in triggering the acceleration of Mg^{++} transport, Group A should have produced a more concentrated urine Mg^{++} during the 6-hour period than Group B. As can be seen in Table III, however, Group B produced the more concentrated urine Mg^{++} ($P < 0.02$). Since the external medium was the same for both groups, the cue for Group B to produce high urine Mg^{++} could not have come from the external medium during the 6-hour period. Furthermore, because the passive water flux in Group B was inward and in Group A was outward, the volume of primary urine formed should be higher in Group B than in Group A, again, assuming a filtration process. It is interpreted that the rate of Mg^{++} transport was indeed responsible for the difference between Groups A and B with respect to urine Mg^{++} concentration, a condition caused by the preliminary treatment in the dilute and concentrated sea water. There is evidence, then, that the rate of Mg^{++} transport is elevated when the salinity of the external medium is increased. Although the experiment was designed to maintain constant concentrations of blood Mg^{++} for both groups, it can be seen that the mean blood Mg^{++} concentration of Group B was higher than that of Group A ($P < 0.02$). Also, the blood osmotic concentration of Group B was, by design, higher than that of Group A ($P < 0.001$). Therefore, high blood Mg^{++} and/or osmotic concentrations possibly triggered the acceleration of Mg^{++} transport.

Group C was treated as follows in an attempt to lower the blood Mg^{++} concentration to that of Group B, but to elevate the blood osmotic concentration above that of Group B. First, the crab was immersed in 158% sea water (82 mM/l. of Mg^{++}) for 18 hours; (2) then the bladder was drained and rinsed with isosmotic perfusion fluid; (3) the crab was reimmersed in 158% sea water for an additional 6 hours when the bladder was again drained, rinsed as before, and (4) the crab was reimmersed in 158% Mg^{++}-free sea water for 6 hours after which the urine was sampled for Mg^{++} analysis. In the above procedure initial exposure to 158% sea water containing natural amounts of Mg^{++} was for the purpose of elevating the blood osmotic concentration by prolonged exposure to hypersaline water; transfer to 158% Mg^{++}-free sea water for the brief period was to maintain high blood osmotic

concentrations, but to reduce the blood Mg^{++} concentration to approximately the level achieved in Group B.

As seen in Table III the mean urine Mg^{++} concentration of Group C is higher than that of Group B $(P < 0.02)$; mean blood Mg^{++} concentrations for the two groups are not significantly different, but the mean blood osmotic concentration of Group C is considerably greater than that of Group B $(P < 0.001)$. It might seem that high blood osmotic concentration triggers the acceleration of Mg^{++} transport. However, values in Table III are terminal and while the mean blood Mg^{++} values for Groups B and C were essentially the same, it is likely that they changed during the 6-hour period when the sampled urine was being formed. On the other hand, as pointed out above, 6-hour urine samples from crabs immersed in 158% sea water have the same concentrations of Mg^{++} whether or not Mg^{++} is present in the medium. There does seem to be some evidence that the osmotic concentration of the blood gives the cue for setting the rate of Mg^{++} transport into the bladder.

Attempts were made to lower blood Mg^{++} further while maintaining high blood osmotic concentrations by prolonged immersion in Mg^{++}-free, hypersaline water. However, individual responses to such treatment were too variable (probably due to different rates of blood Mg^{++} depletion) to permit adequate resolution. On the other hand, blood Mg^{++} concentrations could be elevated while maintaining the blood osmotic concentration constant. Thus, crabs removed from 100% sea water were injected with 0.5 ml. of isosmotic $MgCl_2$ (360 mM/l.) after the bladder was evacuated and rinsed with isosmotic perfusion fluid. The crabs were reimmersed in 100% sea water for 6 hours after which the urine and blood were sampled for Mg^{++} analysis. Thus, the mean blood Mg^{++} (19 cases) was 20.6 mM/l. (S.D., 6.55) and the mean urine Mg^{++} (12 cases) was 158 mM/l. (S.D., 31.4). The mean blood Mg^{++} was significantly higher $(P < 0.001)$ than the mean value 10.0 mM/l. reported for normal crabs immersed in 100% sea water by Gross (1959); the mean (6-hour) urine Mg^{++} was significantly higher than the 6-hour urine Mg^{++} (54.5 mM/l.) reported above for crabs with empty bladders immersed in 100% sea water $(P < 0.001)$. Six crabs treated in the same manner but injected with 0.5 ml. of isosomotic perfusion fluid rather than $MgCl_2$ had a mean urine Mg^{++} of 52.8 mM/l. (S.D., 20.6) which was also significantly less than the value for the Mg^{++} treatment $(P < 0.001)$. Since the injected $MgCl_2$ was isosmotic with the blood, the critical factor in elevating the urine Mg^{++} appears to be the concentration of blood Mg^{++}. There is evidence, therefore, that the rate of Mg^{++} transport into the bladder is influenced by the concentration of Mg^{++} in the blood.

It is concluded that the concentration of urine Mg^{++} in *Pachygrapsus* is determined by: (1) the length of time urine is retained in the bladder, and (2) the rate of transport for Mg^{++} into the bladder. Factors which influence the rate of Mg^{++} transport are: (a) the concentration of blood Mg^{++}, and (b) possibly the osmotic concentration of the blood.

There is no evidence that osmotic or Mg^{++} concentrations of the medium directly influence the rate of Mg^{++} transport. Neither is there evidence that the Mg^{++} flux or the direction of passive water flux directly influences the rate of Mg^{++} transport.

DISCUSSION

There is now convincing evidence that urine first entering the bladder of *Pachygrapsus* has a low concentration of Mg^{++} but a high concentration of Na^+. In time

the urine Mg^{++} concentration increases and the urine Na^+ concentration decreases (Figs. 2, 3 and 4). This probably is accomplished, in part, by a direct Na^+-Mg^{++} exchange across the bladder membranes.

The mean Na^+ concentration change/Mg^{++} concentration change, 1.64, observed in solutions of NaCl or $MgCl_2$ introduced into bladders of crabs kept out of the water (Table II) is compatible with this scheme. Yet, as shown above, when isosmotic perfusion fluid was used instead of NaCl or $MgCl_2$ the ratio was only 1.15, a value that approximates the ratio derived from differences in means for Na^+ and Mg^{++} that occur with time in Figure 3. This conflict may be related to the large Na^+ gradient between blood and urine created by the introduction of pure solutions of NaCl or $MgCl_2$ into the bladder. If the membranes were permeable to Na^+ and Mg^{++} but far less permeable to Cl^-, the rapid diffusion of Na^+ down the gradient across the membranes would necessitate a rapid Mg^{++} exchange because of the low Cl^- permeability. On the other hand, with the slow transport of Mg^{++} that normally occurs into the urine, the probability would be higher that a given Mg^{++} ion could be accompanied by Cl^- ions, thus reducing the necessity of Na^+ exchange for electro-chemical balance and therefore reducing the value for Na^+ concentration change/Mg^{++} concentration change.

Should Cl^- move with Mg^{++}, then an osmotic increase would be caused in the urine, and this would result in an influx of water. Yet, the efflux of exchanged Na^+ would reduce the osmotic concentration of the urine, thus effecting an efflux of water. Since inulin U/B values (Table I) are not less than unity, it is unlikely that net increases in bladder fluid are caused by the inward movement of Cl^- with the transported Mg^{++}.

These data then suggest that during the normal processing of urine in the bladder, there is a direct exchange of Na^+ for the Mg^{++} that is secreted into the bladder, but also, there is some movement of Cl^- with the Mg^{++}, but not in sufficient amounts to cause a net gain of water in the bladder.

Riegel and Lockwood (1961) observed increases in the urine Mg^{++} concentration of *Carcinus* and decreases in urine Na^+ concentration with time as the crab was kept out of water. The increase in Mg^{++} concentration was attributed to Mg^{++} secretion and water withdrawal. However, these authors discounted a direct Na^+-Mg^{++} exchange mechanism because during the test period (*e.g.*, 96 hours) the fall in urine Na^+ concentration (90 mM/l.) seemed too small to account for the rise in urine Mg^{++} concentration (103 mM/l.) on the basis of electro-chemical balance. Now, this might suggest that a direct Na^+-Mg^{++} exchange was not the only process involved, but it does not rule out such a mechanism, for as pointed out above, electro-chemical balance could be achieved both by Na^+-Mg^{++} exchange and Cl^- movement. Besides, Riegel and Lockwood point out that there is water withdrawal from the urine and in such a situation, the movement of Na^+ from urine to blood would be partially obscured by water withdrawal which would increase the concentration in the urine. On the other hand, the apparent movement of Mg^{++} from the blood to the urine would be exaggerated by water withdrawal increasing the Mg^{++} concentration. In end effect, withdrawal of water would reduce the ratio, Na^+ concentration change/Mg^{++} concentration change, below that anticipated.

The wide range of urine Mg^{++} concentrations observed in *Pachygrapsus* (Fig. 1) can be explained largely by the fluctuations of concentration occurring in individual

crabs (Fig. 2) which as indicated above probably reflect the periods of bladder evacuation.

A distinction should be made between the concentration of urine Mg^{++} and the actual net excretion of Mg^{++}; the rate of Mg^{++} transport into the bladder which as indicated above can be varied to meet the load, would, of course, influence both of these, but where there is a prolonged retention of urine in the bladder (e.g., in a crab immersed in hypersaline water), resulting in high urine Mg^{++}, the steep Mg^{++} gradient between blood and urine would likely counteract the effect of accelerated transport. There is no evidence of a good correlation between the ability of a crab to concentrate Mg^{++} in its urine and its ability to regulate Mg^{++} in the blood (Gross, 1964). Gross and Marshall (1960) produced evidence that *Pachygrapsus* loses more Mg^{++} when immersed in 50% sea water than when immersed in 150% sea water even though the urine Mg^{++} of crabs in the dilute medium was only one-sixth the concentration of that for the crabs in hypersaline media. This is interpreted to mean that although the active rate of transport for Mg^{++} into the bladder may have been less for crabs in the dilute medium than in a hypersaline medium, rapid water influxes in the former precluded retention of urine in the bladder, permitting no time for the buildup of a Mg^{++} gradient, thus resulting in less diffusion of Mg^{++} from the urine back to the blood and consequently a greater net transport of Mg^{++} into the urine and to the outside.

It has been shown for *Carcinus* (Webb, 1940) and for *Cancer* (Gross, 1964) that increased Mg^{++} in the medium is reflected in higher urine Mg^{++} concentrations. Such was not shown for *Pachygrapsus* by Gross and Marshall (1960) even though the blood Mg^{++} concentration was elevated by the treatment. It is apparent now that *Pachygrapsus* retains urine in its bladder for a period during which time the urine Mg^{++} concentration is built up (Figs. 2 and 3). Such a phenomenon would shroud the effect of accelerated transport of Mg^{++} if the experiment were initiated on crabs with full bladders. Thus, a crab immersed in hypersaline Mg^{++}-free sea water will appear to concentrate urine Mg^{++} as if the ion were present in high concentrations in the external medium. In this situation Mg^{++} will continue to be pumped into a full bladder probably already containing a high concentration of Mg^{++}. If urine is not evacuated, the Mg^{++} concentration will elevate to a maximum level determined by the osmotic concentration of the isosmotic blood and urine and probably by the magnitude of the Mg^{++} gradient between blood and urine, which, in turn, will depend on the rate of Mg^{++} transport into the urine. Until bladder evacuation occurs no Mg^{++} will be lost by this route and decreases in blood Mg^{++} caused by transport of this ion into the urine could be offset by diffusion of Mg^{++} from the urine back into the blood. Data in Table II show that Mg^{++} can move from urine to blood. Also, if the transport of Mg^{++} involves a direct exchange with Na^+ as the evidence above suggests, the Na^+ concentration gradient may also limit the concentration of Mg^{++} in the urine.

It was only by measuring the Mg^{++} concentration in urine first entering the bladder that the influence of blood Mg^{++} on the rate of Mg^{++} transport could be shown in the present investigation. In the cases of *Carcinus* and *Cancer* where high Mg^{++} concentrations in the medium are reflected in high urine Mg^{++} concentrations when crabs with full bladders are used (Webb, 1940; Gross, 1964), the urine probably is held only briefly in the bladder, no time being permitted to

elevate the Mg^{++} concentration and consequently not obscuring the influence of blood Mg^{++} on the urine concentration of this ion. Gross (1957) produced evidence that the exoskeleton of *Pachygrapsus* is less permeable than that of *Cancer*. Greater water fluxes would be expected in highly permeable animals which, in turn, would not hold urine in the bladder for long; the concentrations achieved for urine Mg^{++} would be expected to be low compared with a relatively impermeable animal. This invites measurement of water fluxes in an array of crabs to determine if the rate of water turnover is related to the maximum concentrations of Mg^{++} achieved in the urine.

Obviously, precise measurements of urine flow would allow quantitative evaluation of the assertions made here. However, meaningful values for urine flow and the consequent ion losses would have to be made under conditions where evacuation of urine from the nephropore was allowed to proceed in a natural manner. Gross and Marshall (1960) have calculated urine flow in *Pachygrapsus* in various salinities from mean urine Mg^{++} concentrations and mean Mg^{++} losses to the medium. Since average values were used, relationships between urine Mg^{++} concentrations that fluctuate in individuals (Fig. 2) and Mg^{++} losses could not be resolved.

In view of the evidence produced above, direct catheterization would, by draining the bladder, deprive it of its normal renal function and probably give spurious values, for urine flow and ion loss. Experiments designed to measure the natural flow of urine in *Pachygrapsus* are in progress, but reliable data have not yet been obtained.

The urinary bladder of *Pachygrapsus* clearly is more than an organ of storage. Although the anatomical details of the bladder are not described, exploratory studies reveal it to be a highly complex, lobed structure similar to those described for other brachyurans in the review by Balss (1944) where histological evidence suggests a secretory function of the bladder wall.

Summary

1. The concentration of urine Mg^{++} in immersed specimens of *Pachygrapsus* is independent of the Mg^{++} influx as well as the concentration of Mg^{++} in the medium. It is, however, a function of the salinity of the medium.

2. Low U/B values for inulin indicate that water withdrawal has little effect in causing the high urine Mg^{++} concentrations and Mg^{++} U/B values observed in *Pachygrapsus*.

3. Repetitive samplings of urine from individual crabs immersed in 100% sea water reveal that the urine Mg^{++} concentration fluctuates with time, varying as much as three-fold in a single crab. This is not believed to be due to fluctuations in the Mg^{++} transport mechanism.

4. The wide range of urine Mg^{++} concentrations observed in the field can be explained chiefly on the basis of fluctuating urine concentrations in individuals rather than on large variations in the ability to concentrate Mg^{++}. ·

5. There is evidence that the membranes of the bladder transport Mg^{++} from blood to urine, and the concentration of Mg^{++} attained in the urine of *Pachygrapsus* depends on the length of time that urine is held in the bladder. Thus, hypo-regulating crabs immersed in hypersaline water having a small water influx will hold urine in the bladder sufficiently long to build up the Mg^{++} concentration. Hyper-regulating crabs in dilute sea water with a large water influx release urine too frequently to permit Mg^{++} buildup. This explains how the urine Mg^{++} concentration can be independent of the Mg^{++} concentration in the medium, but is a function of the salinity of the external medium.

6. Fluctuating urine Mg^{++} concentrations in crabs are believed to indicate periods of bladder evacuation, low Mg^{++} following evacuation and high Mg^{++} preceding evacuation.

7. There is evidence that when Mg^{++} is transported into the urine through the bladder wall, electro-chemical balance is achieved by direct exchange with Na$^+$, but also by some movement of Cl$^-$ with the Mg^{++}. Such a mechanism is compatible with the observed decreases in urine Na$^+$ concentration accompanying increases in urine Mg^{++} concentration.

8. Crabs treated with the Na$^+$ transport inhibitor ouabain can concentrate Mg^{++} in the urine. Thus, there is no evidence that Mg^{++} transport is coupled to the active transport of Na^{++}.

9. Mg^{++} transport from blood to urine is more rapid when the crab is immersed in high salinities than when immersed in low salinities. The mechanism controlling the rate of Mg^{++} transport seems to be triggered directly by the Mg^{++} concentrations in the blood and possibly by the blood osmotic concentration.

10. The concentration of Mg^{++} attained in the urine of a crab does not necessarily indicate the relative ability to excrete Mg^{++}. It is suggested that permeability of the animal to water determines the rate of water turnover and therefore the rate of bladder evacuation. This, in turn, limits the period during which Mg^{++} can be accumulated in a given volume of urine.

11. Direct catheterization of *Pachygrapsus* would be expected to deprive the bladder of its normal renal function, thus giving spurious values for urine flow and ion losses.

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